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Identification of Type I Receptors for Osteogenic Protein-1 and Bone Morphogenetic Protein-4*

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Bone morphogenetic proteins (BMPs) are multifunctional proteins, structurally related to transforming growth factor-β (TGF-β) and activin. TGF-β and activin exert their effects by forming heteromeric complexes of type I and type II serine/threonine kinase receptors. We have previously identified a series of type I serine/threonine kinase receptors, termed activin receptor-like kinase (ALK)-1 to -6. ALK-5 is a TGF-β type I receptor, whereas ALK-2 and ALK-4 are activin type I receptors. Here we investigated the binding of proteins in the BMP family to ALKs. In transfected COS cells, the binding of osteogenic protein (OP)-1 and BMP-4 to certain ALKs was observed in the absence of type II receptors, and their binding was increased after co-transfection of a BMP type II receptor from *Caenorhabditis elegans*, DAF-4. OP-1 bound to ALK-2 and ALK-6 efficiently, and to ALK-3 less efficiently, whereas BMP-4 bound to ALK-3 and ALK-6 efficiently. Similarly, OP-1 bound to ALK-2, ALK-3, and/or ALK-6 in various nontransfected cell lines, although the binding profiles were different between different cell types. BMP-4 bound to ALK-3 in MC3T3-E1 osteoblasts and human foreskin fibroblasts. These results suggest that ALK-3 and ALK-6 are type I receptors for OP-1 and BMP-4; in addition, ALK-2 is a type I receptor shared by activin and OP-1, but not by BMP-4.

Bone morphogenetic proteins (BMPs)[1] are a family of multifunctional proteins, which were originally identified as proteins that initiate bone and cartilage formation in ectopic extraskeletal sites *in vivo* (reviewed in Ref. 1). Several proteins belong to the BMP family, including BMP-2–6 (2, 3) and osteogenic proteins (OP) 1 and 2 (4, 5). BMP-2, BMP-4, and OP-1 (also termed BMP-7; Ref. 3) are 32–36-kDa glycosylated proteins with dimeric structures (2, 6–8). BMP-2 and -4 are highly similar to each other in their structures (92% amino acid sequence identity), whereas they are more distantly related to OP-1 (58–60% amino acid sequence identity). BMPs have various biological effects on different cell types. They stimulate alkaline phosphatase activity and collagen synthesis in osteoblasts, proteoglycan synthesis in chondroblasts (9), chemotaxis of monocytes (10), and differentiation of neural cells (11, 12). Members in the BMP family also have important roles in morphogenesis of the embryo (13).

BMPs belong to the transforming growth factor-β (TGF-β) superfamily, encompassing, *e.g.*, TGF-βs, activins and inhibins, and Müllerian inhibiting substance. TGF-βs and activins exert their effects through binding to specific cell surface receptors (reviewed in Ref. 14). Among several receptors for TGF-βs and activins, the type I receptors of about 53 kDa (15–21) and type II receptors of about 75 kDa (22–25) are serine/threonine kinase receptors, which are shown to be indispensable for signal transduction (26, 27). Type II receptors bind ligands, but they cannot transduce signals in the absence of type I receptors, whereas type I receptors cannot bind ligands in the absence of type II receptors.

The receptors for BMPs have not been fully characterized (24, 28). Recently, the DAF-4 protein, a transmembrane serine/threonine kinase obtained from *Caenorhabditis elegans*, was shown to bind BMP-2 and BMP-4 and to form a 100-kDa cross-linked complex (29). The DAF-4 protein appears not to require other proteins for binding of BMPs, and its structure is more similar to the activin type II receptor (ActR-II) and TGF-β type II receptor (TβR-II), than to the type I receptors, indicating that DAF-4 is a type II receptor for BMP. Thus far, BMP receptors of mammalian origins have not been identified.

A series of novel serine/threonine kinase receptors, activin receptor-like kinases (ALK) 1 to 6, have recently been identified by us (15, 17, 30) and other investigators (18, 20, 31). The sizes of ALKs are 53–58 kDa, which are similar to the reported sizes of the type I receptors. ALK-5 has recently been shown to be a signaling type I receptor for TGF-β (TβR-I; Refs. 15–17), whereas ALK-2 (17–21) and ALK-4 (17) have been shown to be activin type I receptors (ActR-I and ActR-IB, respectively). The ligands for ALK-1, -3, and -6 are yet to be identified. It is also possible that ALK-2, -4, and -5 are receptors shared with the other proteins in the TGF-β superfamily. Therefore, we have systematically investigated whether ALK-1–6 can serve as receptors for OP-1 and BMP-4.

EXPERIMENTAL PROCEDURES

Cell Culture—COS-1 cells, mink lung epithelial cells (Mv1Lu), and AG1518 human foreskin fibroblasts were obtained from American Type Culture Collection. U-1240 MG human glioblastoma cells (32) were obtained from Bengt Westermark, and Tera-2 teratocarcinoma cells (clone 13) (33) were from W. Engström and C. F. Graham. Tera-2 cells (33), MC3T3-E1 cells (34), and ROS 17/2.8 rat osteosarcoma cells (35) were cultured as described. The other cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics.

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[1] The abbreviations used are: BMP, bone morphogenetic protein; ActR, activin receptor; ALK, activin receptor-like kinase; OP, osteogenic protein; TGF-β, transforming growth factor-β; TβR, TGF-β receptor.

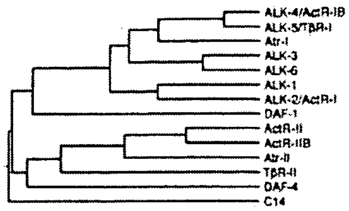

FIG. 1. **Comparison of the structures of serine/threonine kinase receptors.** A phylogenetic tree was prepared based on the amino acid sequence similarities between the kinase domains of serine/threonine kinase receptors. DAF-1 and DAF-4 are from *C. elegans* (29, 38), whereas Atr-II and Atr-I, type II and type I receptors for activin, respectively, are from *Drosophila* (39, 40). The other serine/threonine kinase receptors are from mammals. ALK-1 is also reported as TSR-I (20) and R3 (16). ALK-2 is reported as Tsk-7L (18), SKR-1 (31), and R1 (16). ALK-4 and ALK-5 are reported as R2 and R4, respectively (16). C14 is suggested to be a receptor for Müllerian inhibiting substance, based on the expression profile (41).

Transfection of cDNA—Transient expression plasmids of ALK-1–6 and *daf-4* were generated by subcloning into the pSV7d expression vector (36) or into the pcDNA I expression vector (Invitrogen). For transient transfection, COS-1 cells were transfected with 10 μg each of plasmids by a calcium phosphate precipitation method using a mammalian transfection kit (Stratagene), following the manufacturer's protocol. One day after transfection, the cells were used for the affinity labeling and cross-linking experiments.

Recombinant Proteins and Radioiodination—Recombinant human OP-1 and BMP-4 were obtained as described by Sampath *et al.* (8) and Hammonds *et al.* (6), respectively. Iodination of OP-1 and BMP-4 was performed according to the chloramine T method as described by Frolik *et al.* (37), but chloramine T was added two times instead of three times for the iodination of OP-1.

Binding, Affinity Cross-linking, and Immunoprecipitation—Binding and affinity cross-linking using disuccinimidyl suberate (Pierce Chemical Co.) were performed as previously described (15). Cell lysates obtained by affinity cross-linking were immunoprecipitated using antisera against ALKs as previously described (17). Rabbit antisera against ALK-1–6 were made against synthetic peptides corresponding to the intracellular juxtamembrane parts (17). Samples were then analyzed by SDS-gel electrophoresis using gradient gels consisting of 5–12% or 5–10% polyacrylamide. The gels were fixed and dried and then subjected to autoradiography or analysis using a PhosphorImager (Molecular Dynamics).

RESULTS

A phylogenetic tree based on the similarities between the kinase domains of serine/threonine kinase receptors cloned from different sources is shown in Fig. 1. ALK-1–5 are from human (15, 30), whereas ALK-6 is from mouse (17). Mouse ALK-6 is most similar to human ALK-3, but cloning and sequence analysis of mouse cDNA for ALK-3 indicate that mouse ALK-6 is different from mouse ALK-3.[2] ALK-1–6 are structurally more similar to each other than to the type II receptors for TGF-β and activin, as described previously (17, 30). DAF-4 is, on the other hand, more similar to the type II receptors for TGF-β and activin (29). ALK-5 is a TGF-β type I receptor, whereas ALK-2 and ALK-4 are activin type I receptors.

In order to investigate whether ALKs may act as type I receptors for members in the BMP family, cDNAs for ALKs were transfected into COS-1 cells and tested for the binding of ^{125}I-OP-1 and ^{125}I-BMP-4 in the presence or absence of the *daf-4* cDNA. Since the cross-linked complexes were difficult to visualize because of high background, samples were immunoprecipitated by antisera against each of the six ALKs. When ALK cDNAs were co-transfected with the *daf-4* cDNA, binding of OP-1 to ALK-2/ActR-I and ALK-6 was seen (Fig. 2). ALK-3 was also found to bind OP-1 weakly. In the absence of DAF-4, OP-1 bound to ALK-6, but less efficiently than in the presence of DAF-4. Very weak binding of OP-1 to ALK-2/ActR-I was also observed without co-transfection of *daf-4* cDNA, but other ALKs including ALK-3 did not bind OP-1 in the absence of DAF-4 (data not shown). In contrast to the binding of OP-1, BMP-4 bound to ALK-3 and ALK-6 but not to ALK-2/ActR-I in the presence of DAF-4 (Fig. 2). Binding of BMP-4 to ALK-3 and -6 was also observed in the absence of DAF-4 but less efficiently. The sizes of the cross-linked complexes were slightly higher for ALK-3 than for ALK-2 and -6, consistent with its slightly larger size (17). Type II receptor-like complexes of about 95 kDa were co-immunoprecipitated with ALK-6 and ALK-2/ActR-I. Multiple components of 140–250 kDa could also be co-immunoprecipitated with certain of the ALKs (Fig. 2). The binding of OP-1 and BMP-4 could be competed with excess amounts of unlabeled OP-1 (data not shown). These results suggest that ALK-2, -3, and -6 can serve as type I receptors for BMPs. Notably, however, in contrast to TGF-β and activin, they can weakly bind the ligands without co-transfection of type II receptor cDNAs.

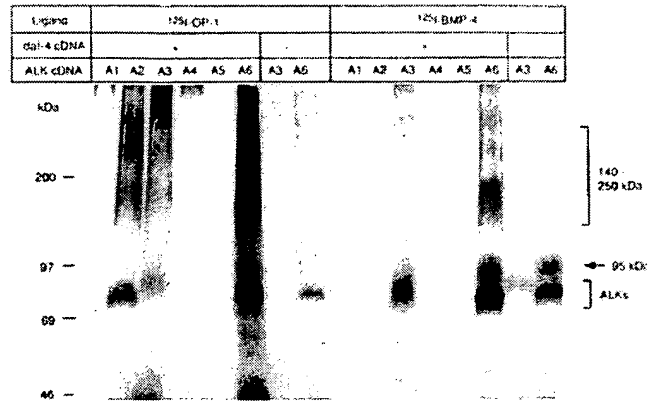

FIG. 2. **Ligand binding studies using COS cells transfected with ALK cDNAs.** Binding of ^{125}I-OP-1 and ^{125}I-BMP-4 to COS cells transfected with ALK cDNA together or not with the *daf-4* cDNA was investigated. Cross-linked complexes were immunoprecipitated by the specific antisera against each ALK and analyzed by SDS-gel electrophoresis and autoradiography. *A1* to *A6* represent ALK-1 to ALK-6.

We have shown previously that many ALKs bind TGF-β1 and activin A in the presence of the respective type II receptors, when they are overexpressed in COS cells. However, in nontransfected cells with normal receptor levels only certain ALKs showed any demonstrable binding of TGF-β1 or activin A (17). Although the binding of ^{125}I-OP-1 and ^{125}I-BMP-4 to transfected COS cells appeared to be more specific than that of ^{125}I-TGF-β1 and ^{125}I-activin A (Fig. 2), it was important to investigate whether ALKs also serve as BMP receptors in nontransfected responsive cell lines.

MC3T3-E1 is a well characterized osteoblastic cell line, which responds to OP-1 and BMP-4 by induction of alkaline phosphatase activity[2] (28). When the cells were affinity-labeled using ^{125}I-OP-1, cross-linked complexes of about 75 kDa were seen, which were immunoprecipitated by the ALK-2/ActR-I antiserum (Fig. 3*A*). In contrast, ^{125}I-OP-1 cross-linked complexes to Mv1Lu cells and U-1240 MG glioblastoma cells were immunoprecipitated by ALK-2/ActR-I and ALK-3, as well as ALK-6 antisera (Fig. 3*A*). Mv1Lu cells are known to express ALK-4 and ALK-5 (17), but cross-linked complexes with ^{125}I-OP-1 were not precipitated by antisera against these receptors. Cross-linked complexes using ^{125}I-OP-1 in Tera-2 teratocarcinoma were immunoprecipitated only by the antiserum to ALK-2/ActR-I (Fig. 3*A*), and those in AG1518 human foreskin fibroblasts were precipitated only by ALK-3 antiserum (data not

[2] P. ten Dijke, H. Yamashita, C.-H. Heldin, and K. Miyazono, unpublished observations.

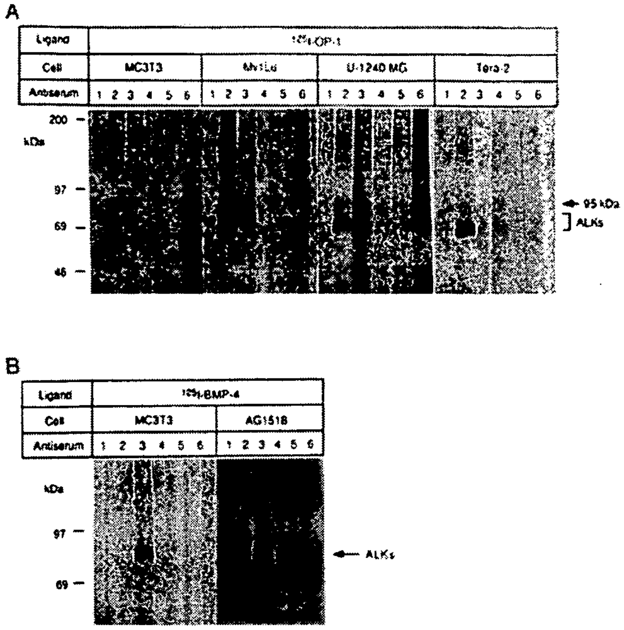

FIG. 3. **Identification of receptors for OP-1 and BMP-4 in nontransfected cells.** Binding and affinity cross-linking using ^{125}I-OP-1 to MC3T3-E1 mouse osteoblasts, Mv1Lu mink lung epithelial cells, U-1240 MG human glioblastoma cells, and Tera-2 human teratocarcinoma cells (*A*), or using ^{125}I-BMP-4 to MC3T3-E1 mouse osteoblasts and AG1518 human foreskin fibroblasts (*B*), were followed by immunoprecipitation using the ALK antiserum. *Antisera 1–6* represent those for ALK-1–6. Samples were analyzed by SDS-gel electrophoresis and autoradiography using a PhosphorImager analysis or Fuji x-ray films.

TABLE I
Binding of ALKs to OP-1 and BMP-4 in different cell types

Cell lines	Binding of OP-1	Binding of BMP-4
Mouse osteoblasts (MC3T3-E1)	ALK-2	ALK-3
Mink lung epithelial cells (Mv1Lu)	ALK-2, -3, -6	Not done
Human glioblastoma (U-1240 MG)	ALK-2, (-3), -6[a]	Not done
Human teratocarcinoma (Tera-2)	ALK-2	(–)
Human foreskin fibroblasts (AG1518)	(ALK-3)	ALK-3
Rat osteosarcoma (ROS17/2.8)	ALK-2, (-3)	Not done

[a] Parentheses indicate very weak binding.

shown). Cross-linking of ^{125}I-OP-1 to ROS17/2.8 rat osteosarcoma cells yielded immunoprecipitated components by ALK-2/ActR-I and ALK-3 antisera (data not shown). Similar to the results obtained by transfection of cDNA into COS cells, binding of OP-1 to ALK-3 appeared to be less efficient than to ALK-2/ActR-I and ALK-6 in most cell types. Type II receptor-like components of about 95 kDa as well as high molecular mass complexes of 140–250 kDa, co-immunoprecipitated with certain of the ALKs in Mv1Lu cells, U-1240 MG cells, and Tera-2 cells (Fig. 3*A*). In conclusion, ALK-2/ActR-I, ALK-3, and ALK-6, which were shown to bind ^{125}I-OP-1 in the COS cell system, can serve as OP-1 type I receptors in nontransfected cells; however, the binding profiles were different between different cell types (Table I).

Receptors for BMP-4 in nontransfected cells were also investigated. Cross-linked complexes of ^{125}I-BMP-4 bound to MC3T3-E1 osteoblasts and AG1518 human foreskin fibroblasts were immunoprecipitated only by the ALK-3 antiserum (Fig. 3*B*). On the other hand, cross-linking of ^{125}I-BMP-4 to Tera-2 cells did not yield any immunoprecipitated components by antisera against ALKs (data not shown).

DISCUSSION

In the present study, we show that among the six ALKs analyzed, ALK-3 and ALK-6 are receptors for OP-1 and BMP-4. In addition, OP-1 but not BMP-4 bound ALK-2, which has been shown previously to be a type I receptor for activin (17, 20, 21). Binding of these ALKs was facilitated in the presence of DAF-4, suggesting that these ALKs and DAF-4 act as type I and type II receptors for the BMPs, respectively. Although both OP-1 and BMP-4 have bone and cartilage inducing activity *in vivo*, their structures are not closely related. The present study showed that they have similar but not identical binding properties to type I serine/threonine kinase receptors.

Binding of TGF-β1 or activin A to ALKs was dependent on the presence of the type II receptors. ALK-5/TβR-I, but not other ALKs, binds TGF-β1 only very weakly without co-transfection of TβR-II cDNA into COS cells, whereas none of the ALKs bind activin A in the absence of ActR-II (17). Weak binding of ALK-5/TβR-I to TGF-β1 in the absence of TβR-II is most likely due to TβR-II endogenously expressed in COS cells. Binding of ALKs to BMPs observed in the transfected COS cells (Fig. 2) appeared to be less dependent on the type II receptors compared to the TGF-β and activin receptor systems. Thus, COS cells may possibly express small numbers of endogenous BMP type II receptors, which allow ALKs to bind ligands without transfection of *daf-4* cDNA. Alternatively, OP-1 and BMP-4 may bind type I receptors in the absence of type II receptors. DAF-4 may also affect the binding to type I receptors indirectly, *e.g.* by decreasing the turnover of the type I receptors, and thus increase their expression levels in the transfected cells. It is also possible that certain ALKs may form heteromeric complexes upon the addition of BMPs; however, co-transfection of combinations of two different cDNAs of ALK-2/ActR-I, ALK-3, and ALK-6 did not significantly increase the binding of ^{125}I-OP-1 or ^{125}I-BMP-4, compared to transfection of single cDNAs (data not shown).

We could observe co-immunoprecipitated type II receptor-like complexes of 95 kDa in COS cells transfected with cDNAs for ALK-6. Such components were also observed after co-transfection of *daf-4* cDNA with ALK-6 or ALK-2/ActR-I cDNA, but not with ALK-3 cDNA (Fig. 2). Cross-linked complexes containing DAF-4 was not clearly observed in the transfected COS cells, probably due to inefficient cross-linking of DAF-4 to ^{125}I-labeled ligands or inefficient co-immunoprecipitation with ALKs. Type II receptor components were also observed in certain cell types (Fig. 3*A*), indicating that these cells express endogenous type II receptors on the cell surface.

Complexes of approximately 140–250 kDa could be seen in COS cells transfected with ALK and *daf-4* cDNAs, as well as in certain nontransfected cells. Since the 140–250-kDa complexes were brought down by the ALK antisera, the complexes are likely to contain ALKs or proteins that form complexes with ALKs.

In nontransfected cells, we showed that OP-1 bound ALK-2/ActR-I, ALK-3, and ALK-6, but the binding profiles of OP-1 to ALKs were different in different cell types (Fig. 3*A* and Table I). This may be in part due to the different expression profiles of different ALKs (17, 30). Northern blot analysis using different human and mouse tissues revealed that ALK-2/ActR-I is ubiquitously expressed and ALK-3 was observed in skeletal muscle and certain other tissues, whereas ALK-6 was found only in brain and lung.

The binding of OP-1 and BMP-4, as well as TGF-β and activin (17), to ALKs is summarized in Fig. 4. Based on the

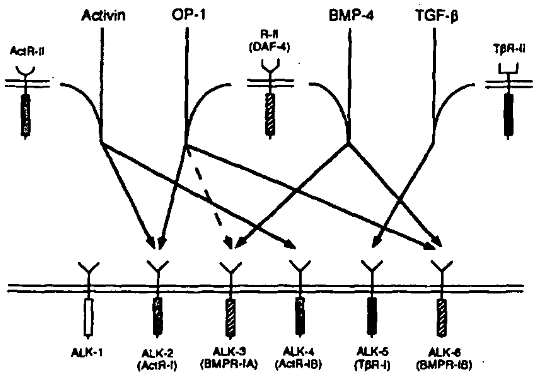

FIG. 4. **Schematic illustration of the binding of TGF-β, activin, OP-1 and BMP-4 to ALKs.** Among six ALKs, TGF-β binds to ALK-5 with a high affinity in the presence of TβR-II (15–17), whereas activin binds to ALK-2 and ALK-4 with high affinities in the presence of ActR-II (17). OP-1 and BMP-4 bind to ALK-3 and ALK-6, although the binding of OP-1 to ALK-3 was less efficient than that of BMP-4. ALK-3 and ALK-6 are tentatively designated BMPR-IA and BMPR-IB, respectively, in this figure. OP-1 also binds to ALK-2, which is shared by activin. Binding of OP-1 and BMP-4 to ALKs is facilitated by DAF-4, a BMP type II receptor obtained from *C. elegans.* Mammalian BMP type II receptors have not been identified. It is possible that different type II receptors form complexes with ALK-3 and -6 and with ALK-2 for the binding of OP-1 and BMP-4. Ligand(s) for ALK-1 has not been identified.

binding of OP-1 and BMP-4, ALK-3 and ALK-6 are designated BMPR-IA and BMPR-IB, respectively (Fig. 4). These designations are tentative, pending determination of the roles of ALK-3 and ALK-6 in signaling by BMPs. It should be also noted that ALK-2/ActR-I is shared by activin and OP-1, but not by BMP-4. ALK-3 and ALK-6 are structurally very similar to each other, whereas ALK-2/ActR-I is less related (Fig. 1). These results suggest that ALK-3 and ALK-6 transduce similar intracellular signals, which may be important for biological effects induced by members of BMP family. The present data revealed that DAF-4 bound OP-1 and facilitated the binding to ALK-3 and -6, as well as to ALK-2/ActR-I (Fig. 2). However, it is possible that different type II receptors form complexes with ALK-3 and -6, and with ALK-2/ActR-I in mammalian cells. Future studies will be aimed at the identification of mammalian BMP type II receptors and the mode of complex formation between different type I and type II receptors and different BMPs. It will also be important to elucidate whether different ALKs transduce different downstream signals upon the addition of OP-1, BMP-4 and other proteins in the BMP family.

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